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EXPOSURE OF MICROORGANISMS TO SIMULATED EXTRATERRESTRIAL SPACE ECOLOGY

by

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In order to survive exposure to the stresses of outer space environments a microorganism either must possess an inherent resistant capability or be protected. Certain of these stress parameters have been simulated in ground test systems. It is the intention of the authors to describe certain of the experimental requirements necessary for adapting ultra-high vacuum equipment to microbiological studies as well as certain results obtained from these studies conducted over the last year and one-half. This investigation was conducted by National Research Corporation, Cambridge, Mass. in conjunction with MIT.

A number of studies have examined the effects of high and ultra-high vacuum on microorganisms at ambient temperatures. Prince and Bakanauskas (1958) and Prince (1960) noted that freeze-dried spores of Aspergillus niger, A. flavus, Bacillus globigii and B. mycoides survived up to 32 days in vacuums ranging from 1×10^{-5} to 5×10^{-7} torr.

Portner et al. (1961) exposed B. subtilis, A. niger, A. terreus and Penicillium citrinum to vacuum in the ultra-high range (3.6×10^{-10} torr) at ambient temperature for 5 days and concluded that these organisms would survive the vacuum of outer space. Brueschke, Suess, and Willard (1961) subjected spores of B. subtilis, A. niger, A. terreus and P. citrinum to vacuums of 1×10^{-6} to 6×10^{-9} torr. P. citrinum was not recovered, but the other test organisms were recovered after 10 days at which time a vacuum of 8×10^{-8} torr was achieved. None of the organisms were viable after 30 days, at 1.2×10^{-8} torr. Subsequent publications by Morelli, Fehlner, and Stembridge (1962a, b) demonstrated, in agreement with those of Prince and Portner et al., that B. subtilis var. niger spores survived 35 days at 10^{-8} torr at room temperature with little or no destruction.

This investigation is concerned with the viability of spores of 5 test organisms and those present in soils after exposure to a wide range of temperatures, or to gamma radiation, while under ultra-high vacuum. The vacuums employed were from 10^{-8} to 10^{-10} torr, corresponding to an altitude of from approximately 200 to 400 miles.

The microbial assay methods and procedures employed in this study have been described elsewhere (Davis, Silverman, and Keller, 1963) and will not be discussed in detail. Spores of B. megaterium, B. subtilis var. niger, B. stearothermophilus, Clostridium sporogenes and A. niger were the test organisms. These organisms were, after suitable heat-activation where

necessary, impinged upon glass fiber filter paper, dried at 45°C for 2½ hours and maintained in a desiccator over silica gel until they were inserted in the vacuum apparatus. The duration of an experiment was 5 days, this period including the time required for final temperature and vacuum attainment.

One local soil and one obtained from the Mohave desert were also subjected to temperature-vacuum experiments. The more moist local soil was predried overnight at 37°C and sieved through a 48 mesh screen. In order to prevent excessive pressures in the vacuum chamber when the system was heated, the amount of soil for any experiment was limited to 5 1-g portions which were distributed in petri dishes. The control and vacuum chamber exposed soils were assayed for heterotrophic mesophilic aerobes, anaerobes, actinomycetes, molds, and in certain experiments thermophiles.

Vacuum-Temperature Equipment

For most of the experiments at elevated temperatures the apparatus described in Fig. 1 was used. By careful placement of the resistance heaters a temperature gradient within the chamber was obtained, each shelf being subjected to a different temperature zone as illustrated in Fig. 2. It is also seen that vacuum is altered by the extent of outgassing from the metal surfaces. The higher the final chamber temperature the longer the delay in achieving the ultra-high vacuum range. In subsequent experiments employing temperatures in excess of 120°C, smaller chambers were used and the sample was subjected to a single temperature. By cooling the chamber walls, so as to minimize outgassing, vacuums in excess of 10^{-8} torr were routinely reached at temperatures in excess of 150°C.

For low temperatures, of -110 to -190°C, a 5 cubic foot vacuum chamber was modified so that the glass fiber filters containing the spores were in direct contact with a copper heat transfer system. Due to the low temperatures, outgassing was minimized and the final vacuum reached was lower, in the 10^{-10} torr region (Fig. 3).

Results and Discussion

The five test organisms showed marked differences in their ability to survive exposure to elevated temperatures while under ultra-high vacuum (Table 1). Appreciable percentages of all of the test organisms were recovered after exposure to temperatures

up to and including 60°C. Above this temperature only B. subtilis var. niger and A. niger were notably resistant to this combination of stresses. A. niger appeared to be the most resistant of the test organisms; an extremely small portion of the exposed spores were still viable after exposure to 107°C but not to 120°C. In comparison, no viable spores of B. subtilis var. niger were recovered after being heated to 100°C in an ultra-high vacuum for 4 to 5 days. Exposure to -110 and -190°C and ultra-high vacuum did not appear to be lethal to the test spores, which is not surprising since these conditions are somewhat comparable to the lyophilization technique so commonly employed for preserving microbial cultures.

Spores exposed to 90°C and atmospheric pressure presented a different survival pattern (Table 2). Of the five test organisms, only two, B. subtilis var. niger and much smaller numbers of B. megaterium, were recovered after 24 hours. B. subtilis var. niger was recoverable after incubation for 5 or more days at this temperature. A. niger, which was the most resistant test organism in ultra-high vacuum, did not survive 24 hours exposure at 90°C.

With the exception of B. subtilis var. niger, ultra-high vacuum appeared to protect B. stearothermophilus, B. megaterium, C. sporogenes and especially A. niger to prolonged exposure to elevated temperatures in the vicinity of 90°C. There appears to be a threshold temperature where this protective effect occurs since a comparison of recovery of these test spores at 60°C (Table 3) at atmospheric pressure and in ultra-high vacuum indicates that at this temperature ultra-high vacuum is not protective. Considering the large differences in resistivity between B. subtilis var. niger, A. niger and the remaining three test spores in ultra-high vacuum it is conceivable that we are dealing with either an ability to prevent the vacuum distillation of essential cell constituents or a capability for maintaining sufficient cell integrity to enable a cell to resume growth upon rehydration. For some reason A. niger loses this property when dried at 90°C at atmospheric pressure.

Recovery data obtained by colony counts is often not indicative of what occurs at the individual cell level. In order to determine some aspects of the extent of damage to the spores after being heated in an ultra-high vacuum, germination of B. subtilis var. niger was investigated by phase contrast microscopy. The results of a microscopic assay of B. subtilis var. niger for the percentage germinated, ungerminated and those

spores capable of undergoing outgrowth are presented in Fig. 4 to 8 and in Table 4. It is seen that after exposure to temperatures up to 69°C for 5 days almost all of the spores either germinated or produced vegetative cells after 2 hours incubation at 37°C on a tryptone-glucose extract agar medium. Above 83°C outgrowth, even after 24 hours incubation ceased, and the percentage germination decreased with increasing temperatures until after exposure to 120°C, 87% of the spores remained refractile (Fig. 8).

Soils

Two types of soils, a garden soil obtained from the Cambridge vicinity and another from the Mohave desert, were examined at various temperatures at ultra-high vacuum. In Table 5 the garden soil showed quantitative recovery of mesophilic aerobes, mesophilic anaerobes, and actinomycetes but no molds at temperatures up to 100°C. At higher temperatures a qualitative recovery procedure was employed, and mesophilic aerobes, molds and a large number of actinomycetes were recovered.

The higher recovery of anaerobes at 100°C over that at lower temperatures is the result of heat shocking the soil prior to plating--a treatment which had not influenced the recovery of C. sporogenes after vacuum-heat treatment.

One aspect of interest in this study is a comparison of the thermal resistivity of microorganisms exposed to elevated temperatures in ultra-high vacuum to those exposed at atmospheric pressure. Of the test spores A. niger was extremely resistant at temperatures of 90°C and higher in an ultra-high vacuum but showed extreme susceptibility to 90°C at atmospheric pressure (Table 2). This indicated that a screening procedure for heat resistant organisms, employing an oven at atmospheric pressure, might not be capable of predicting whether certain organisms, which, due to a unique physiological response, might be capable of surviving drying at ultra-high vacuums but not at atmospheric pressure. That this is indeed the case is shown by the following experiment.

Fifty isolates from desert soil exposed to 120°C and vacuum for 4.5 days were returned to the vacuum chamber on glass filters. Seven cultures were recovered which included six colorless, punctiform sporeformers and one amber, butyrous organism, as yet uncharacterized. Only one of these, a colorless punctiform bacillus colony type, survived 120°C for 3 hours at atmospheric pressure.

Protection by soil is indicated by the results shown in Table 6 for Mohave desert soil. Forty-three typical isolates which survived 170°C for 4-5 days at 6 X 10^{-9} torr were also screened at 120°C for 3 hours at atmospheric pressure. Only five bacteria survived this treatment. Future experiments will determine their resistivity in ultra-high vacuum at elevated temperatures.

Bruch, Koesterer and Bruch (1962) had previously noted that mesophilic sporeformers in soil displayed a higher resistance to dry heat as compared to the isolates placed on filter paper strips.

Irradiation of Ultradried Organisms

Many organisms are more sensitive to ionizing radiation when they are irradiated in air than when they receive the same radiation dose under anoxic conditions. This oxygen effect is known to occur in aqueous suspensions for a variety of organisms, and has been reported for X-irradiated dry spores of B. megaterium (Powers, 1961).

In our investigation, the gamma radiation resistance of extremely dry spores which had been in ultra-high vacuum was compared with the resistance of similar spores which had been stored over silica gel. It was also possible to determine whether vacuum per se was lethal. Spores in ultra-high vacuum were at pressures in the 10^{-9} mm Hg range. At these low pressures, the spores are extremely dry. Volatile external spore constituents as well as internal components which could pass the spore coat barrier were certainly being removed from the spores during the several days the spores were in vacuum.

Washed spores of B. stearothermophilus, B. megaterium, and C. sporogenes were dried on glass fiber filter circles at 45°C, and stored overnight over silica gel. About one million spores were pipetted onto each filter. The filters were mounted on a stainless steel wire support which was inserted into a glass tube fitted with an ultra-high vacuum valve assembly and an ultra-high vacuum gauge. It was possible to transport each assembly to the Cobalt-60 irradiator so that the tube contents were maintained under ultra-high vacuum. A hot-filament ultra-high vacuum gauge attached to each tube made it possible to determine pressures before and after the tubes were irradiated.

An ultra-high vacuum assembly is shown in Fig. 9. Each assembly is about 21 inches in length and is attached to a vacuum chamber having a pumping arrangement identical to that shown in Fig. 1.

Spores were maintained in ultra-high vacuum under continuous pumping for 4.5-5.5 days. The valves were then closed, and the 7 tubes were detached from the chamber for irradiation experiments and for plating as vacuum controls.

The tube-vacuum valve system is shown in detail in Fig. 10 with two tubes positioned in a rack ready for irradiation. Normally one tube was irradiated under vacuum and the other tube opened to admit dry air and then closed before irradiation.

Manifold System

Pressures in the individual tubes when loaded with six filters rose from 10^{-9} torr range before valve closure to about the middle 10^{-8} torr range after valve closure. The entire sample assembly consisting of the ultra-high vacuum valve, the gauge and the sample tube were transported to a submerged pool type Co^{60} facility for gamma irradiation at an intensity of 5000 rad per minute.

In each experiment, spores were irradiated while in vacuum, in dry air after vacuum treatment, and after storage over silica gel at atmospheric pressure. Filters were then blended, and appropriate dilutions were plated on suitable media. Membrane filters were used where necessary. A portion of the B. stearothermophilus blend was heated in a boiling water bath for 30 minutes before these spores were plated. With the exception of B. stearothermophilus, which was incubated at 55°C, all organisms were incubated at 30°C for 1 or 2 days as necessary for maximal counts.

The results are presented in Fig. 11 and Table 7. The radiosurvival curves shown in Fig. 11 indicate that B. megaterium is the most resistant and C. sporogenes the least resistant of the three organisms irradiated in the dry state. One question of interest is whether these dried spores are more or less radioresistant than those normally hydrated. The radioresistivity of the stock suspension of spores of B. stearothermophilus irradiated in phosphate buffer saturated with nitrogen was greater than for those spores irradiated in the dry state. A better comparison would be between dried spores and hydrated spores placed on glass

filter discs and this data is now being collected. Nevertheless it is of pertinence that B. stearothermophilus spores were appreciably less radioresistant after ultradrying. Elimination of a portion of the indirect effects by removal of water was not of a sufficient magnitude to overcome what appears to be an inherently decreased radioresistivity. Other investigations indicate that bacterial spores may be less radioresistant in the dry state (Pepper, Buffa and Chandler, 1956), and Alper (1961) in her review indicated that moisture content may be a physiological factor in radioresistivity rather than a dose modifying factor such as air.

The presence of air does, in fact, modify the dried spores radioresistivity (Table 7). Column A is the percent survival of the organisms at various doses under vacuum and was illustrated in Fig. 11. The values in columns B and C demonstrate that subsequent exposure to air of the spores dried in ultra-high vacuum increased their sensitivity to gamma radiation. Moderate drying over a desiccant and subsequent irradiation in air (column D) resulted in a radioresistivity greater than that noted for the ultradry spores (column B) also exposed to air and of equivalent survival to that noted for ultradried spores irradiated in vacuum (column A).

Tallentire (1958) noted that spores of B. subtilis dried on kaolin under low vacuum (10^{-3} torr) for 6 hours experienced an oxygen effect when irradiated with gamma rays. He stated that oxygen contributes to direct rather than to indirect radiation effects on the spore. The data in Table 7, obtained at a much higher vacuum for considerably drier spores, appears to substantiate the observation of Tallentire and also that of Powers (1961) for the oxygen dependent damage to spores of B. megaterium.

Undoubtedly the interrelationships between moisture content, its effect on the physiological organization of the spore and the composition of the gaseous phase are complex and require further clarification.

These experiments are continuing, and it is intended to compare the radioresistivities of microorganisms in various hydrated states at different oxygen levels in addition to combining heat with irradiation on ultradried spores.

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TABLE 1

Per Cent Survival of Spores Following Exposure

To Ultrahigh Vacuum at Various Temperatures for Five Days

Organism	-190°C	-110°C	25°C	53°C	60°C	88°C	100°C	107°C	120°C
B. stearothermophilus	118	67	67	14	1.2	0.001	-	-	-
B. subtilis var. niger	93	119	113	63	40	0.01	NG*	NG*	-
B. megaterium	88	90	98	2.6	0.7	<0.001	-	-	-
C. sporogenes	125	83	88	<1	0.1	<0.01	-	-	-
A. niger	126	83	98	-	25	0.2	0.03	G**	NG*

* No Growth (NG): $< 2 \times 10^{-6}$ per cent

** Growth (G): $< 6 \times 10^{-3}$ per cent

TABLE 2

Survival of Spores at 90°C and Atmospheric Pressure

Organism	1 day	2 days	3 days	4 days	5 days	6 days	7 days
B. stearothermophilus	0	-	-	-	-	-	-
B. subtilis var. niger (Expt. A)	48	26.5	11	3.1	0.55	0.08	0.002
B. subtilis var. niger (Expt. B)	32.6	12.6	4.2	0.46	0.09	-	-
B. megaterium	0.005	0	-	-	-	-	-
C. sporogenes	0	-	-	-	-	-	-
A. niger	0	-	-	-	-	-	-

TABLE 3

Per Cent Survival of Dry Spores During Five Days at 25°C and 60°C in Vacuum and at Atmospheric Pressure

Organism	Atmospheric Pressure				Ultrahigh Vacuum		
	Days at 60°C			Days at 25°C	Days at 60°C		Days at 25°C
	0	4	5	5	0	4-5	4-5
	spores/filter	%	%	%	spores/filter	%	%
B. stearothermophilus	1.6×10^6	30	35	56	1.3×10^5	1.2	67
B. subtilis var. niger	1.8×10^6	67	72	78	1.8×10^5	40	113
B. megaterium	1.5×10^6	80	87	87	1.8×10^5	0.7	98
C. sporogenes	1.6×10^6	48	62	106	3.0×10^5	0.1	88
A. niger	0.7×10^6	61	9	120	1.3×10^5	25	98

TABLE 4

Germination of B. subtilis var. niger Spores and Refractility Following Exposure to Ultrahigh Vacuum at Various Temperatures for Five Days

Vacuum Chamber Temperature-°C	TGE Agar			1.5% Agar	
	Non-refractile %	Refractile %	Outgrowth %	Non-refractile %	Refractile %
25°Control	11	1	88	9	91
20°	21	3	77	6	94
40°	23	2	75	15	85
52°	58	1	41	13	87
69°	85	0	15	12	88
83°	98	2	< 0.1	17	83
90°	92	8	< 0.1	-	-
100°	89	11	0	5	95
107°	64	36	0	5	95
120°	13	87	0	-	-

TABLE 5

Per Cent Survival of Organisms in Garden Soil

Exposed to Ultrahigh Vacuum at Various Temperatures for Five Days

	40°C	52°C	69°C	90°C	100°C	110°C	120°C	170°C
Mesophilic Aerobes	30.4	15.4	7.8	0.15	0.3	0.03	0	+
Mesophilic Anaerobes	1.3	<0.002	<0.002	<0.002	0.06	0.02	0	0
Molds	100	100	60	2.0	-	0	0	+
Actinomycetes	60.5	38	25.8	14.2	12.6	0.8	+[1]	+

[1] - Qualitative - Growth occured in recovery broth from one gram of soil

TABLE 6

Microbiological Analysis of Mohave Desert Soil Exposed to 170°C for 4.5 Days at 6×10^{-9} torr

	Organisms per gram		
	Bacteria	Molds	Actinomycetes
Mesophilic Aerobes	22	14	4
Thermophilic Aerobes	1	0	0
Mesophilic Anaerobes	9	-	0
Thermophilic Anaerobes	5	-	0

TABLE 7

Per Cent Survival of Spores Irradiated in Vacuum and in Air After Being Dried in Ultrahigh Vacuum

	Dose (rad x 10^3)	A $\frac{IV}{UV}(100)$	B $\frac{IVO}{UV}(100)$	C $\frac{IVO}{IV}(100)$	D $\frac{IA}{UA}(100)$
B. stearothermophilus	100	15	9	59	30
	200	12	1	8.5	6.5
	300	1.3	0.01	0.41	1.8
	500	0.3	*	*	*
B. megaterium	100	86	53	61	61
	200	43	8.3	20	27
	300	9.3	0.47	5	2.7
	400	1.5	0.06	4	1.7
C. sporogenes	100	20	14	74	51
	200	17	0.3	2	19
	300	1.4	*	*	7
	400	0.14	*	*	0.12

I - Irradiated U - Unirradiated O - Opened to Air

V - Vacuum A - Air, stored in desiccator

* - No detectable survivors at this irradiation dose

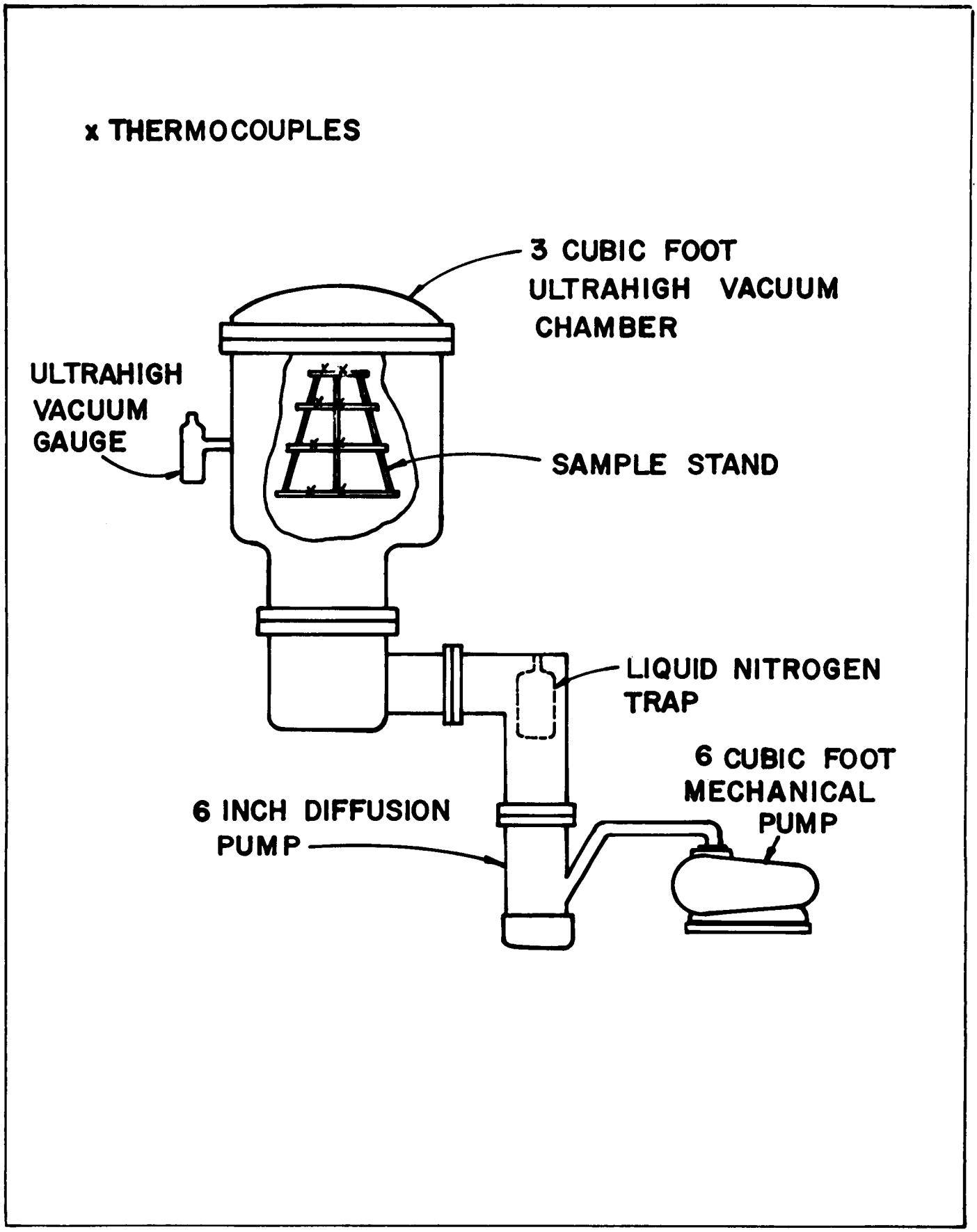


FIGURE 1

ULTRAHIGH VACUUM SYSTEM FOR AMBIENT AND

ELEVATED TEMPERATURE EXPERIMENTS

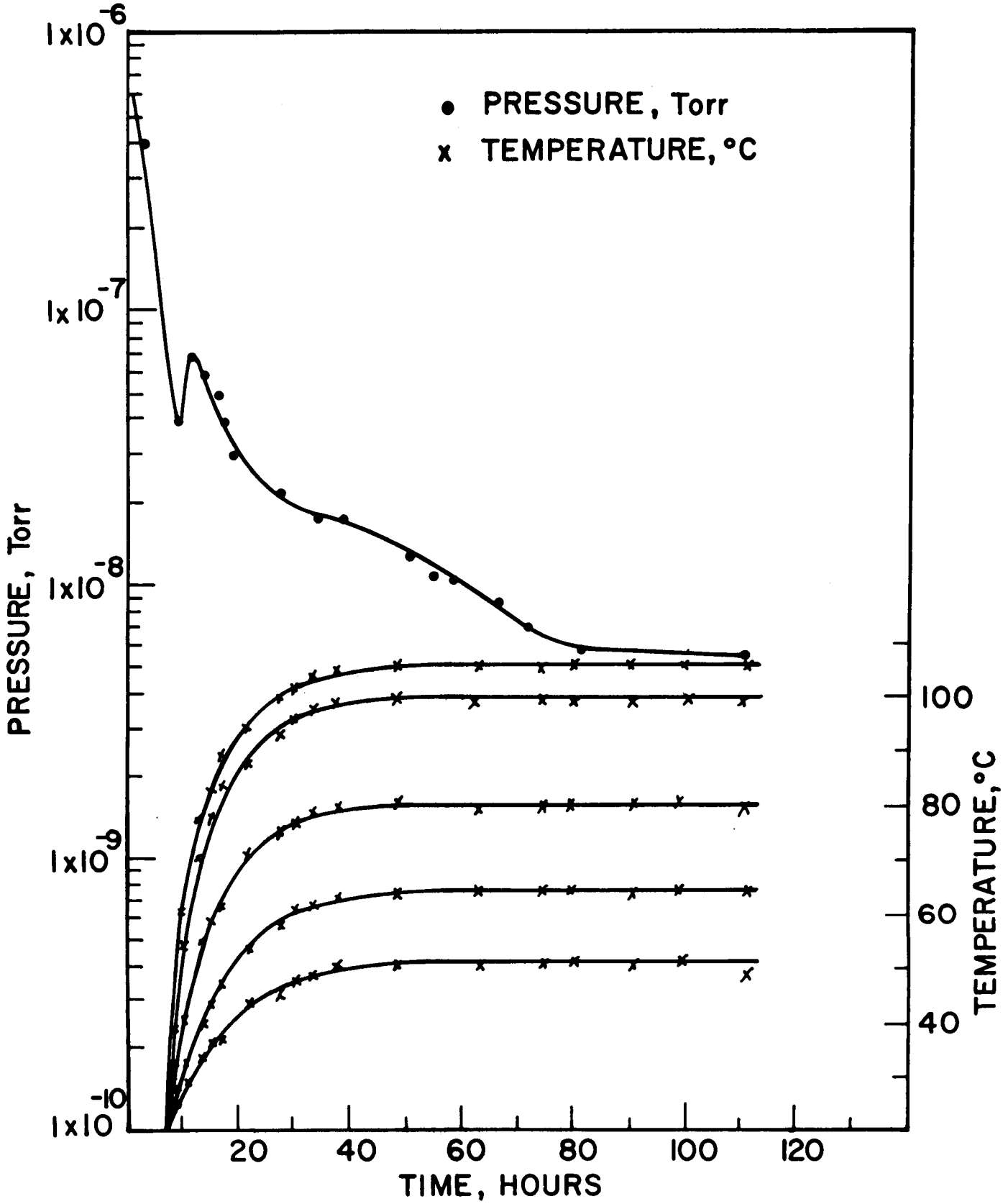


FIGURE 2

THE VERTICAL TEMPERATURE GRADIENT AT FIVE LEVELS

IN AN ULTRAHIGH VACUUM SYSTEM

HEATED BY AN EXTERNAL RESISTANCE HEATER

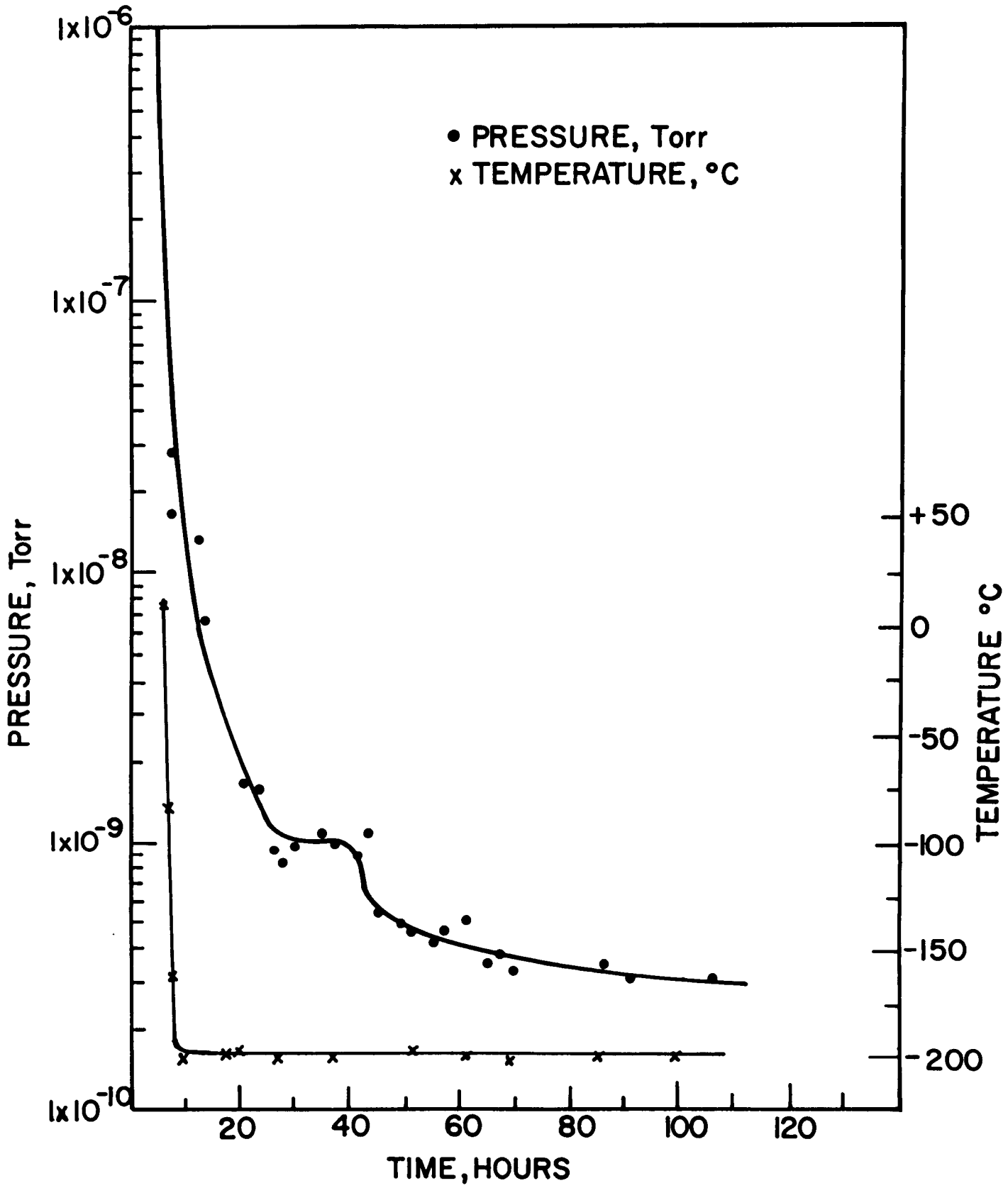
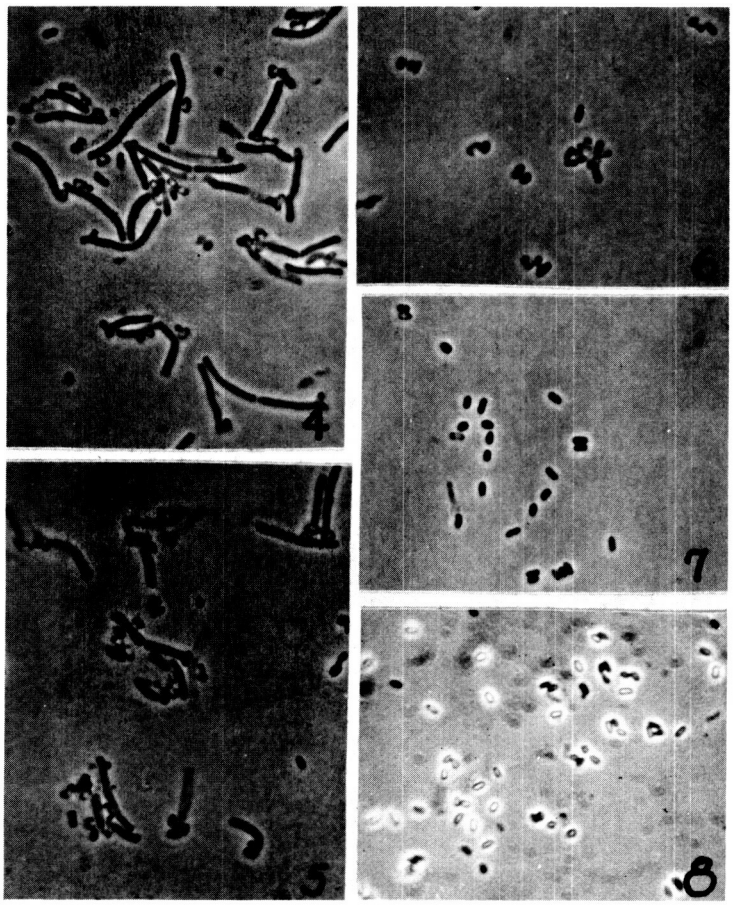


FIGURE 3

PRESSURE-TEMPERATURE-TIME RELATIONSHIPS IN AN ULTRAHIGH VACUUM SYSTEM COOLED WITH LIQUID NITROGEN



FIGURES 4 - 8

B. SUBTILIS VAR. NIGER SPORES EXPOSED TO ULTRAHIGH VACUUM AT VARIOUS TEMPERATURES AND SUBSEQUENTLY INCUBATED AT 37°C ON TRYPTONE GLUCOSE EXTRACT AGAR

Figure 4 - Control not exposed to vacuum; 2 hour incubation

Figure 5 - 40°C; 2 hour incubation

Figure 6 - 69°C; 2 hour incubation

Figure 7 - 83°C; 24 hour incubation

Figure 8 - 120°C; 24 hour incubation

FIGURE 9

A COMPLETED ULTRAHIGH VACUUM ASSEMBLY CONNECTED TO THE MAIN VACUUM CHAMBER USED FOR GAMMA RADIATION OF TEST SPORES

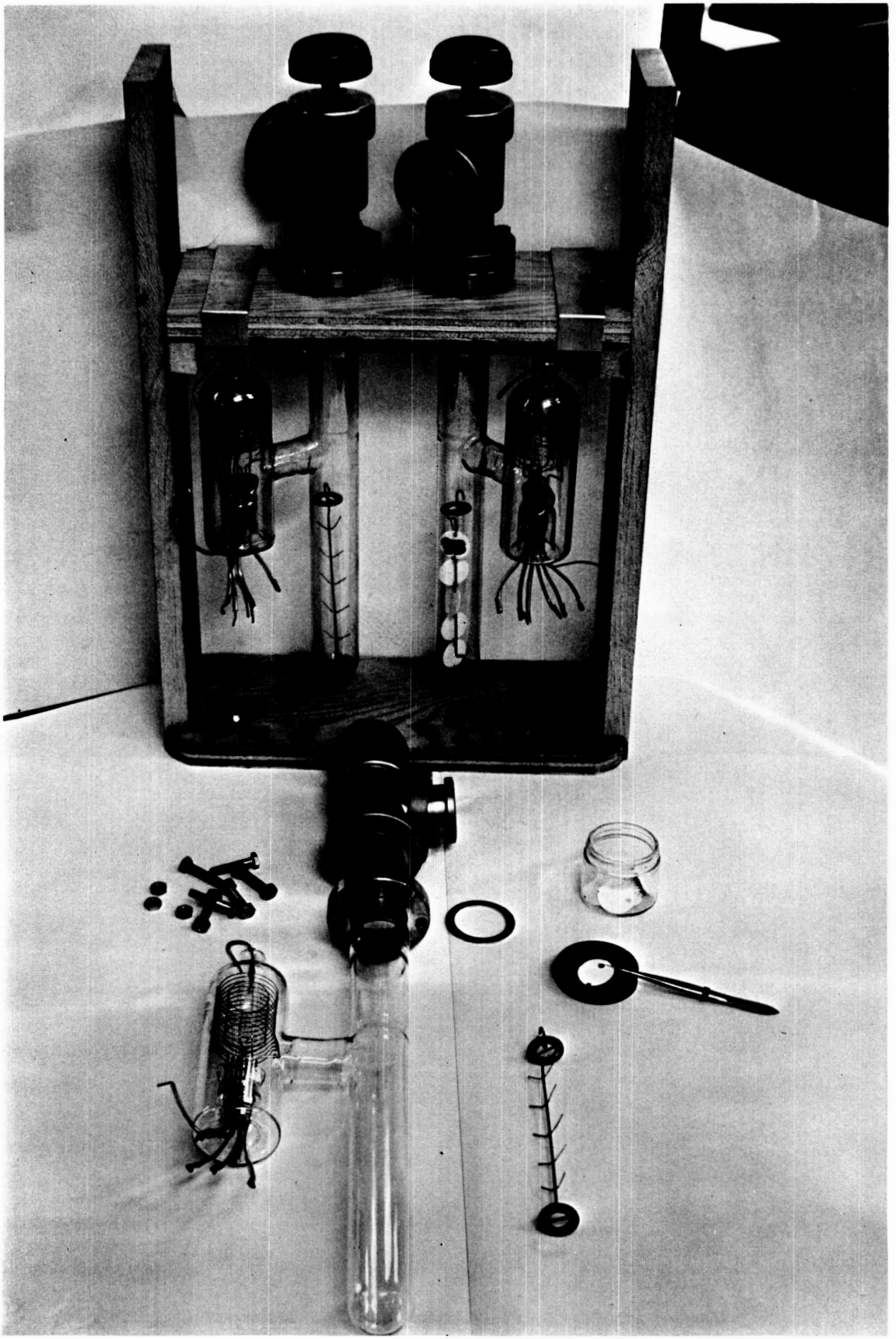


FIGURE 10

THE COMPONENT PARTS OF AN ULTRAHIGH VACUUM ASSEMBLY AND THE FRAME USED FOR EXPOSING THE ASSEMBLY TO GAMMA RADIATION

FIGURE 11
RADIORESISTANCE OF SPORES AFTER DRYING WITH ULTRAHIGH VACUUM AND COMPARISON TO RESISTIVITY OF STOCK SPORES OF BACILLUS STEAROTHERMOPHILUS IRRADIATED IN BUFFER (pH 7.0) UNDER NITROGEN